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Research Article

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PROSER1 mediates TET2 O-GlcNAcylation to regulate DNA demethylation on UTX-dependent enhancers and CpG islands

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DNA methylation at enhancers and CpG islands usually leads to gene repression, which is counteracted by DNA demethylation through the TET protein family. However, how TET enzymes are recruited and regulated at these genomic loci is not fully understood. Here, we identify TET2, the glycosyltransferase OGT and a previously undescribed proline and serine rich protein, PROSER1 as interactors of UTX, a component of the enhancer-associated MLL3/4 complexes. We find that PROSER1 mediates the interaction between OGT and TET2, thus promoting TET2 O-GlcNAcylation and protein stability. In addition, PROSER1, UTX, TET1/2, and OGT colocalize on many genomic elements genome-wide. Loss of PROSER1 results in lower enrichment of UTX, TET1/2, and OGT at enhancers and CpG islands, with a concomitant increase in DNA methylation and transcriptional down-regulation of associated target genes and increased DNA hypermethylation encroachment at H3K4me1-predisposed CpG islands. Furthermore, we provide evidence that PROSER1 acts as a more general regulator of OGT activity by controlling O-GlcNAcylation of multiple other chromatin signaling pathways. Taken together, this study describes for the first time a regulator of TET2 O-GlcNAcylation and its implications in mediating DNA demethylation at UTX-dependent enhancers and CpG islands and supports an important role for PROSER1 in regulating the function of various chromatin-associated proteins via OGT-mediated O-GlcNAcylation.

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Introduction

Enhancers are genomic DNA elements which are able to interact with and activate gene promotors irrespective of their genomic location or orientation, often acting over long genomic distances (Banerji et al, 1981, 1983; de Villiers et al, 1982). Many critical developmental genes are under the regulation of enhancers (Levine, 2010). The histone mark H3K4me1 is highly enriched at active enhancers with significantly lower enrichment at poised/inactive enhancers, whereas H3K4me3 mainly occurs at gene promoters (Heintzman et al, 2007, 2009; Rada-Iglesias et al, 2011). We and others have shown that the epigenetic regulators MLL3 and MLL4 (also known as KMT2C and KMT2D) function as major H3K4 monomethyltransferases on enhancers and are required for enhancer activation during developmental transitions (Herz et al, 2012; Lee et al, 2013; Hu et al, 2013a; Wang et al, 2016a). MLL3 and MLL4 exist in large protein complexes that also contain the H3K27 demethylase UTX (also known as KDM6A) (Agger et al, 2007; Cho et al, 2007; Lee et al, 2007; Mohan et al, 2011; Rickels et al, 2020). Understanding how the MLL3/4 complexes regulate chromatin structure and function to control enhancer activity and transcription is of high importance as *UTX*, *MLL3*, and *MLL4* belong to some of the most frequently mutated genes across a broad spectrum of adult and pediatric cancers and are also mutated in various neurodevelopmental disorders (Huether et al, 2014; Herz, 2016; Bailey et al, 2018; Priestley et al, 2019; Lavery et al, 2020). Thus, identifying the pathways that regulate the recruitment and function of the MLL3/4 complexes at genomic elements will provide a foundational framework for the development of future therapeutic approaches to foster the treatment of multiple human diseases involving the UTX/MLL3/MLL4 axis.

Results

Identification of PROSER1, a novel proline- and serine-rich protein, the DNA demethylase TET2, and the glycosyltransferase OGT as factors that associate with the MLL3/4 complexes

To identify new components that functionally intersect with the MLL3/4 complexes, we purified FLAG-UTX from human embryonic kidney (HEK293) cells. By mass spectrometry (MS) analysis we detected the H3K4 methyltransferases MLL3 and MLL4, and all

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For hMeDIP-seq

Rabbit α-5-hmC (39791; Active Motif), 10 μg per hMeDIP.

Immunoprecipitation (IP)

For large scale IP

Cells at 90–95% confluency from 10 150 mm plates were scraped off with a cell scraper and pelleted by centrifugation. The pellet was washed once with cold DPBS, centrifuged, resuspended in five pellet volumes of Buffer A and incubated on ice for 15 min. After centrifugation, the pellet was resuspended in two pellet volumes of Buffer A. Cytoplasmic lysis was carried out by homogenization with a 15 ml Dounce Tissue Grinder with 15 strokes using a tight pestle (357544; Wheaton). The nuclei were centrifuged for 5 min at 1,500*g* at 4°C and then resuspended in Buffer C and adjusted to a total volume of 5 ml. Nuclear proteins were extracted by incubation on a nutator for 30 min at 4°C and centrifuged for 30 min at 50,000 rpm at 4°C using an ultracentrifuge (rotor SW 55 Ti; Beckman). The protein concentrations of the nuclear extracts were determined by a protein assay (5000006; Bio-Rad) with a spectrophotometer (6133000010; Eppendorf) using disposable cuvettes (0030079353; Eppendorf). Equal total protein amounts were used for all samples of the same IP experiment. Preclearing was conducted with 200 μl packed Protein A/G Plus Agarose beads (sc-2003; Santa Cruz) per IP sample (from 0.8 ml bead slurry) by incubation on a nutator for 1 h at 4°C. After centrifugation for 1 min at 850*g* at 4°C, the precleared supernatant was collected and a small volume was set aside as input. FLAG affinity purifications were carried out with 50 μl packed (100 μl bead slurry) FLAG M2 affinity gel (A2220; Sigma-Aldrich) or FLAG magnetic beads (A36797; Pierce) per IP sample on a nutator overnight at 4°C. On the next day the beads were centrifuged for 1 min at 850*g* at 4°C or separated using a magnetic stand (12321D; Thermo Fisher Scientific). The beads were washed three times with Wash Buffer and one time with Elution Base Buffer. Proteins were eluted by adding 400 μl Elution Base Buffer supplemented with 200 μg/ml FLAG peptide (F3290; Sigma-Aldrich) to the FLAG agarose beads or FLAG magnetic beads followed by incubation with mixing on a nutator for 30 min at 4°C. The eluates including the FLAG agarose beads were transferred onto a Micro Bio-Spin column (89868; Pierce) and collected by centrifugation for 2 min at 850*g* at 4°C. For the FLAG magnetic beads, the elutes were collected by using a magnetic stand. The flow-through (eluates) was saved for further processing.

For small scale IP

Cells at 90–95% confluency from one 100 or 150 mm plate were scraped off with a cell scraper and pelleted by centrifugation. The pellet was washed once with DPBS. Cytoplasmic lysis was carried out by resuspending the cell pellet with 1 ml Buffer A supplemented with 0.5% NP-40 and incubated on a nutator for 5 min at 4°C. The nuclei were centrifuged for 5 min at 1,500*g* at 4°C and then resuspended in Buffer C. If using total cell lysates, the cell pellets were directly lysed in Buffer C supplemented with 0.5% NP-40. Protein extraction was performed by incubation on a nutator for 30 min at 4°C and centrifugation for 30 min at 20,817*g* at 4°C using a table top centrifuge (5430R; Eppendorf). Equal protein amounts were used for all samples of the same IP experiment. For FLAG IPs, 50 μl FLAG magnetic bead slurry (A36797; Pierce) was used. For TET2 IPs, 50 μl Dynabeads Protein G slurry (10004D; Thermo Fisher Scientific) and 5 μg TET2 antibody (A304-247A; Bethyl Laboratories) were used. Incubation was performed on a nutator at 4°C for at least 2 h. After the incubation, the beads were collected with a magnetic stand and subjected to washing. The washed beads were then boiled in 1× SDS Laemmli Buffer for 10 min at 95°C.

Buffer A

10 mM Hepes, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM DTT; and protease inhibitors (P8340; Sigma-Aldrich) (1:200).

Buffer C

20 mM Hepes, pH 7.9; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 10% glycerol; 0.5 mM DTT; and protease inhibitors (P8340; Sigma-Aldrich) (1:200).

Wash Buffer

20 mM Hepes, pH 7.9; 300 mM NaCl; 1.5 mM $MgCl_2$; 10% glycerol; 0.1% Triton X-100; 0.5 mM DTT; and protease inhibitors (P8340; Sigma-Aldrich) (1:500).

Elution Base Buffer

20 mM Hepes, pH 7.9; 100 mM NaCl; 1.5 mM $MgCl_2$; 10% glycerol; 0.05% Triton X-100; 0.5 mM DTT; and protease inhibitors (P8340; Sigma-Aldrich) (1:500).

4× SDS Laemmli Buffer

250 mM Tris, pH 6.8; 50% glycerol; 8% SDS; and 0.1% bromophenol blue. Before using, add 100 μl of β-mercaptoethanol to 900 μl of 4× buffer.

Glycerol gradient fractionation

The eluates of two large scale FLAG affinity purifications of FLAG-UTX from HEK293 cells were combined and brought up to 1 ml by addition of Elution Base Buffer. The eluates were then layered over 10 ml of a 20–50% glycerol gradient and centrifuged for 48 h at 35,000 rpm at 4°C using an ultracentrifuge (rotor SW 41 Ti; Beckman). 33 fractions of ~325 μl each were collected and analyzed by WB for UTX, RBBP5, PROSER1, and TET2.

RNA-seq

RNA was isolated from 3 × 10^6 cells with the RNeasy Mini Kit (74106; QIAGEN) following the manufacturer's instructions with the following alterations. Cells were homogenized in 600 μl RLT buffer (with 2-Mercaptoethanol) and passed through a QIAshredder column (79656; QIAGEN) by centrifugation in a table top centrifuge for 2 min at full speed at RT. The optional step after the second wash with RPE buffer was applied to dry the membrane. For DNA digestion, RNA was eluted with 85 μl H_2O and mixed with 10 μl 10× DNAase buffer and 5 μl DNAse I (M0303S; NEB), then incubated at RT for 20 min. After incubation, RNA was purified by following the "RNA Cleanup" protocol in the RNeasy Mini Handbook. RNA was finally eluted in 50 μl H_2O and concentration determined with a NanoDrop 8000 Spectrophotometer. The following RNA quantification, quality check, strand-specific library preparation, and sequencing were performed by the Hartwell Center at St. Jude Children's Research Hospital as previously described (Mondal et al, 2020).